THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Production of a recombinant form of early pregnancy factor that can prolong allogeneic skin graft survival time in rats

Citation for published version:
Morton, H, McKay, DA, Murphy, RM, Somodevilla-Torres, MJ, Swanson, CE, Cassady, AI, Summers, K & Cavanagh, AC 2000, 'Production of a recombinant form of early pregnancy factor that can prolong allogeneic skin graft survival time in rats', *Immunology & Cell Biology*, vol. 78, no. 6, pp. 603-7. https://doi.org/10.1046/j.1440-1711.2000.00951.x

Digital Object Identifier (DOI):
10.1046/j.1440-1711.2000.00951.x

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Immunology & Cell Biology

Publisher Rights Statement:
© 2000 Australasian Society for Immunology

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

OPEN ACCESS

Download date: 07. May. 2023

Immunology and Cell Biology (2000) **78**, 603–607

Research Article

Production of a recombinant form of early pregnancy factor that can prolong allogeneic skin graft survival time in rats

H MORTON,[1] DA MCKAY,[1] RM MURPHY,[1] MJ SOMODEVILLA-TORRES,[1] CE SWANSON,[1] AI CASSADY,[2] KM SUMMERS[1*] and AC CAVANAGH[1]

[1]*Department of Surgery and* [2]*Centre for Molecular and Cellular Biology, The University of Queensland, Brisbane, Queensland, Australia*

Summary Early pregnancy factor (EPF), an extracellular chaperonin 10 homologue, has immunosuppressive and growth factor properties. In order to carry out more extensive studies on the *in vivo* characteristics of EPF, a recombinant form of the molecule has been prepared. Recombinant human EPF (rEPF) was expressed in *Escherichia coli* using the plasmid pGEX-2T expression system. Potency of rEPF *in vitro* in the rosette inhibition test, the bioassay for EPF, was equivalent to that of native EPF (nEPF), purified from human platelets, and synthetic EPF (sEPF). However, the half-life of activity (50% decrease in the log value) in serum, following i.p. injection, was significantly decreased (3.2 h, compared with nEPF 6.2 days, sEPF 5.8 days). This was thought to be due to modification of the N-terminus of the recombinant molecule inhibiting binding to serum carrier proteins. Because EPF can modify Th1 responses, the ability of the recombinant molecule to suppress allogeneic graft rejection was investigated. Following skin grafts from Lewis rats to DA rats and vice versa, rEPF was delivered locally at the graft site and the effect on survival time of the allografts noted. Results demonstrated that rEPF treatment significantly prolonged skin graft survival time by as much as 55% in stringent models of transplantation across major histocompatibility barriers.

Key words: chaperonin 10, early pregnancy factor, local immunosuppression, recombinant protein, rosette inhibition test, skin graft.

Introduction

Early pregnancy factor (EPF) is a secreted protein with immunosuppressive and growth factor properties. It was first described in mice as a factor appearing in maternal serum within 24 h of fertile mating.[1] Subsequently, it has been shown to be present in all mammalian species studied, persisting for at least the first two-thirds of pregnancy (reviewed by Morton[2]). Like other cytokines and growth factors, EPF has pleiotropic effects.

Early pregnancy factor was first shown to have an immunomodulatory role with the demonstration that it could suppress the adoptive transfer of contact sensitivity to trinitrochlorobenzene from sensitized mice to naïve, syngeneic mice.[3,4] This assay is a measure of the delayed-type hypersensitivity (DTH) response and is evidence that EPF suppresses Th1 responses. Further evidence has been provided by its ability to suppress phytohaemagglutinin-induced stimulation of mouse spleen cells.[2] Early pregnancy factor modulates the DTH response by stimulation of downregulatory factors from T lymphocytes, designated EPF-S_1 and EPF-S_2, from both mouse and human lymphocytes.[5,6] This suggests that EPF can initiate a cascade reaction and that some of the immunomodulatory effects of EPF are downstream from initial EPF binding event.

In addition to immunomodulatory effects, we have also found that EPF plays a role in cell growth and survival. Early pregnancy factor is essential for the embryo during normal development[7,8] and for tissue renewal, as shown in liver regeneration following partial hepatectomy.[9] As well as a role in normal cell proliferation, EPF also acts as an autocrine growth factor for tumour cells.[10]

We have now identified EPF as a homologue of chaperonin 10 (Cpn10),[11] a heat shock protein (hsp) found in mitochondria.[12] Chaperonin 10 and EPF differ in functional targets and mechanisms of action. Chaperonin 10 specifically interacts with chaperonin 60 within the cell to facilitate correct folding of a variety of protein targets,[12] whereas we have shown that EPF has extracellular cytokine-like activities as described earlier. Despite amino acid sequence identity between the human EPF and Cpn10 proteins,[13] recent studies (BH Fletcher *et al.* unpubl. data, 2000) suggest that they are encoded by different genes.

Further studies of the *in vivo* function of EPF have been difficult to undertake, because only insignificant quantities of EPF could be obtained from natural sources. We have now prepared recombinant EPF (rEPF) in *Escherichia coli* and have used this tool to investigate the ability of the recombinant molecule to prolong survival time of allogeneic skin graft in rats. Following skin grafts from Lewis rats, rEPF was

Correspondence: Dr H Morton, The University of Queensland, Department of Surgery, Clinical Sciences Building, Royal Brisbane Hospital, QLD 4029, Australia.
Email: h.morton@mailbox.uq.edu.au
*Present address: KM Summers, Queensland Clinical Genetics Service, Queensland Health, Brisbane, QLD 4029, Australia.
Received 21 March 2000; accepted 25 May 2000.

delivered locally at the graft site to DA rats, and vice versa, and the effect on survival time of the allografts noted.

Materials and Methods

Animals

Inbred male Lewis and DA rats (approximate weight 100 g), inbred mature BALB/c mice and outbred mature male Quackenbush mice were obtained from the Central Animal Breeding House, The University of Queensland. Animals were maintained on a continuous supply of mouse/rat pellets and water, in temperature- (22–26°C) and light- (12 h light, 12 h dark) controlled rooms. Investigations were carried out in accordance with Australian National Health and Medical Research Committee guidelines with ethical clearance from The University of Queensland Animal Experimentation Ethics Committee.

Recombinant EPF

Early pregnancy factor was produced as an in-frame fusion at the C-terminus of glutathione *S*-transferase (GST) using the pGEX-2T (Amersham Pharmacia Biotech, Uppsala, Sweden) expression system. The *Bam*HI–*Eco*RI fragment of pRM3,[13] containing the complete open reading frame of human EPF, was ligated into equivalent sites in pGEX-2T and this construct was used to transform DH5α competent cells. Transformed cells were grown, induced with isopropyl β-D-thiogalactose (IPTG) and lysed using standard conditions.[14] The GST fusion protein was recovered from the cell lysate (6 L original culture) with 10 mL glutathione-Sepharose 4B gel (Amersham Pharmacia Biotech). Following extensive washing of the gel, rEPF was cleaved from the immobilized fusion protein by thrombin (750 units thrombin (T6884; Sigma-Aldrich, St Louis, MO, USA) in 1 mL 0.05 mol/L Tris-HCl pH 8.0/0.15 mol/L NaCl/2.5 mmol/L $CaCl_2$ (Tris/Ca buffer)) and recovered in the supernatant and three subsequent washes of the gel with 3 mL Tris/Ca buffer/wash (sample 1). A control buffer (buffer 1) was prepared, containing 750 units thrombin/10 mL Tris/Ca buffer. The gel was washed with 4 mL high-salt buffer (0.05 mol/L Tris-HCl pH 8.0/2 mol/L NaCl; buffer 2) and supernatant containing rEPF recovered (sample 2). Samples were stored frozen at –30°C and thawed only once. Prior to injection, sample 1 and buffer 1 were diluted in 0.15 mol/L NaCl/1% normal syngeneic rat serum, as appropriate. Sample 2 and buffer 2 (plus 1% rat serum) were exchanged into saline/1% rat serum on a NAP5 column (Amersham Pharmacia Biotech) before dilution.

Protein concentration was estimated by the Bradford method (Bio-rad Protein Assay, Richmond, CA, USA) with bovine serum albumin as the standard. Purity of preparations was determined by SDS-PAGE in 15% midget gels ($10 \times 8 \times 0.75$ mm) using a Tris-HCl/Tris-Tricine discontinuous buffer system. N-terminal amino acid sequencing was performed by the Department of Biochemistry, The University of Queensland, using an Applied Biosystems Model 470A gas-phase sequencer. Electrospray mass spectrometry was performed by the Centre for Drug Design and Development, The University of Queensland, with a PESCIEX API III triple quadripole mass spectrometer.

Native EPF

Native EPF (nEPF) was purified from platelets, harvested from clinically outdated human blood, as previously described.[11] This molecule is N-terminally acetylated. The average yield was 15 µg EPF from approximately 50 L blood.

Synthetic EPF and EPF-derived peptides

Synthetic acetylated and non-acetylated EPF (sAcEPF and sEPF) and bioactive EPF-derived peptides, corresponding to the N-terminus (Ac1–11; AcAGQAFRKFLPL; N-peptide[11]) and an internal amino acid sequence (34–44; EKSQGKVLQAT; I-peptide[11]), were synthesized by stepwise solid-phase techniques.[15] The peptides had an additional cysteine (C) added to the C terminal end to facilitate conjugation to ovalbumin in preparation of antibodies in studies described elsewhere.[16]

Rosette inhibition test: Bioassay for EPF

Early pregnancy factor (0.5 mL, 100 µg/mL in PBS/0.01% (mass/vol.) BSA) was exchanged into 1.0 mL Hank's balanced salt solution/0.01% BSA (HBSS/BSA) on a NAP5 column (final concentration rEPF 50 µg/mL (5 µmol/L)). The EPF-derived peptides (5 µg) were dissolved in 1.0 mL HBSS/BSA (5 µmol/L). Samples were diluted 10-fold in HBSS/BSA from 10^{-5} to 10^{-15} and the rosette inhibition titre (RIT) of each dilution determined using spleen cells from Quackenbush mice, as described previously.[17] The EPF titre (log reciprocal sample dilution) was recorded as the highest dilution of a sample to give a positive result in the assay.

Time course of rEPF in serum following i.p. injection into mice

Following i.p. injection of rEPF (1.0 or 15.0 µg/mouse), nEPF, sAcEPF or sEPF (1.0 µg/mouse), N-peptide or I-peptide (0.1 µg/mouse) in saline/1% BALB/c mouse serum into BALB/c mice, the time course of activity in serum was determined by the rosette inhibition test.[17] One of each of these preparations was administered to a group of three mice and animals were bled from the tail vein at various times after injection (see Fig. 2). Serum was harvested, inactivated at 56°C for 0.5 h, frozen in small volumes at –30°C and thawed once only. Samples were tested in the rosette inhibition test and EPF titre determined. The half-life of EPF activity in serum was defined as the time after injection when serum EPF titre gave a 50% decrease in log value compared with that at 0.25 h.

Allogeneic skin grafts

Skin grafts were exchanged between Lewis and DA rats. Rats were anaesthetized with Nembutal (pentobarbitone sodium, Merial Australia, Parramatta, NSW, Australia; 6 mg/100 g bodyweight), shaved, full-thickness skin (1.3 cm diameter) removed from the abdomen and sutured into a similar-sized defect created in the lateral thoracic region. Each rat received two grafts, one allograft and one isograft (control of technique), one on either side of the thorax. Six rats were grafted in one session, with two Lewis and two DA rats receiving rEPF (1 µg, 5 µg, 20 µg or 70 µg/rat per 12 h in 0.25 mL saline/1% rat serum). One Lewis and one DA rat received vehicle alone. Recombinant EPF or vehicle was delivered with a 29 gauge needle at multiple sites around the graft. Treatment continued for 14 days. Six rats/rEPF dose were grafted in the DA to Lewis series and five rats/rEPF dose in the Lewis to DA series with the exception of groups receiving 70 µg rEPF/dose (two rats/group). Grafts were covered with Sofra-tulle strip (Roussel Laboratories, Uxbridge, UK), followed by Melolin dressing (Smith + Nephew, Clayton, Vic., Australia), held in place with Co-flex cohesive flexible bandage (Andover Coated products, Salisbury, MA, USA). After 7 days, grafts were examined daily for signs of necrosis. Day of rejection was taken as that on which 50% of transplanted skin had undergone necrotic degradation. The sEPF and nEPF were not tested in this assay.

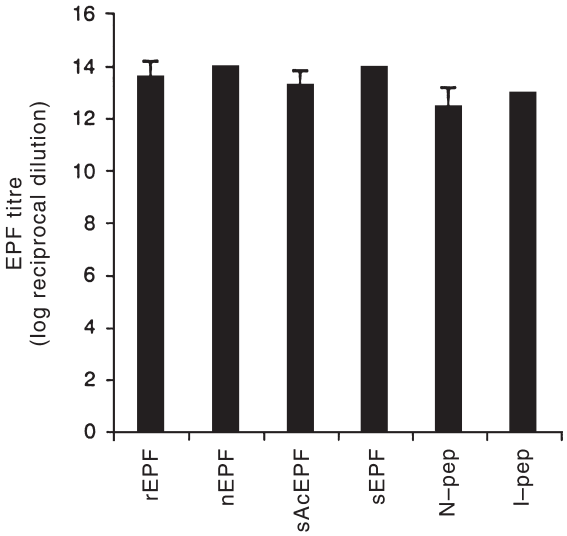

Figure 1 Early pregnancy factor (EPF) titre of different preparations of EPF and EPF-derived peptides. Preparations (5 μmol/L) were diluted 10-fold from 10^{-5} to 10^{-15} and the rosette inhibition titre (RIT) of each dilution determined. The EPF titre (log reciprocal sample dilution) was recorded as the highest dilution of a sample to give a positive result in the assay. rEPF, recombinant human EPF; nEPF, native EPF; sAcEPF, synthetic acetylated EPF; sEPF, synthetic non-acetylated EPF; N-pep, N-terminal peptide; I-pep, internal peptide.

Statistical analysis

Student's *t-test* was used for comparison of paired and unpaired measurements. Time to graft rejection was compared using the logrank statistic. The 0.05 level of significance was used throughout.

Results

Recombinant EPF

Average yield was 0.3–0.4 mg rEPF/L of culture, with the majority of rEPF in sample 1. Both samples 1 and 2 had a high level of purity (85–90% and 90–95%, respectively). N-terminal amino acid sequencing of material, following reversed phase (RP)-HPLC purification, gave the sequence GSMAGQAF, confirming fidelity of the thrombin cleavage reaction and the expected addition of GS from the thrombin cleavage site, as well as the initiating M residue at the N-terminus of the recombinant protein. The molecule is not N-terminally acetylated. Extremely close agreement of determined (11077.0) and predicted (11075.5) mass demonstrated that no unexpected alterations to the protein had arisen in this expression system and confirmed that only a single protein species was present.

Biological potency of rEPF

Potency of the recombinant molecule in the rosette inhibition test (Fig. 1) did not differ from that of the full-length native molecule, the synthetic molecules (sAcEPF, sEPF) or synthetic peptides (N-peptide, I-peptide). Modification of the N-terminus did not affect biological activity of the protein in this assay.

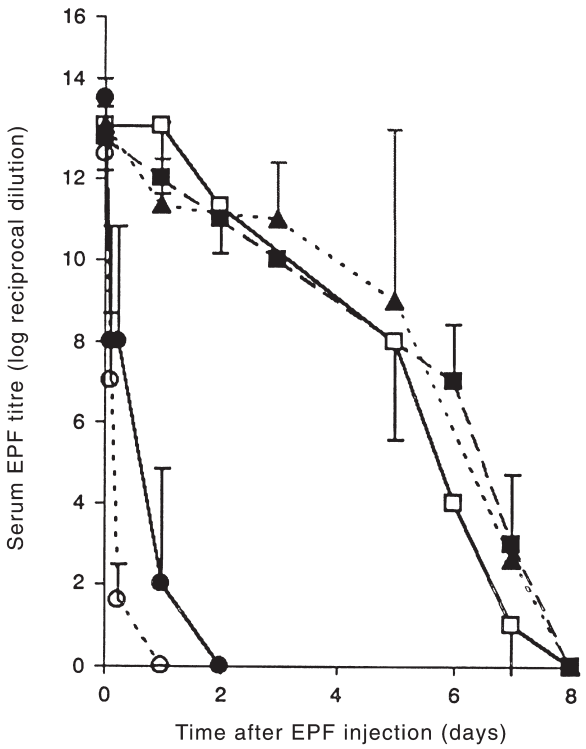

Figure 2 Time course of early pregnancy factor (EPF) activity in serum following injection i.p. of various preparations into BALB/c mice. Recombinant human EPF (1 μg (○) or 15 μg (●)), native EPF (■), synthetic acetylated EPF (□) or synthetic non-acetylated EPF (▲; 1 μg) were injected i.p. into male BALB/c mice (three mice/group). Mice were bled from the tail vein on the days indicated and the EPF titre in serum determined by the rosette inhibition test.

Time course of activity in serum following injection i.p. into mice

In contrast to the above results, the half-life of rEPF activity in serum (3.2 h) was significantly decreased compared with that of nEPF (6.2 days), sAcEPF (5.3 days) and sEPF (5.8 days; $P < 0.001$; Student's *t*-test). Non-acetylation alone was not responsible, because the active half-life of non-acetylated sEPF did not differ from that of the acetylated preparation. It appears that the modified N-terminus of rEPF (additional GSM) markedly shortened the time course of its activity in serum when compared with the unmodified molecules (Fig. 2). This suggestion was supported by the time course of activity of EPF-derived peptides in serum. The N-terminal peptide has an active half-life in serum (7.1 days) not significantly different from that of the native or synthetic molecules, while the I-peptide has an activity (3.0 h) similar to that of rEPF ($P < 0.001$; Student's *t*-test; data not illustrated).

Allogeneic skin grafts

Recombinant EPF significantly prolonged the survival time of skin grafts, both DA to Lewis and Lewis to DA, as shown in Fig. 3. Results showed a bell-shaped dose–response, with high as well as low doses being relatively ineffective. In both

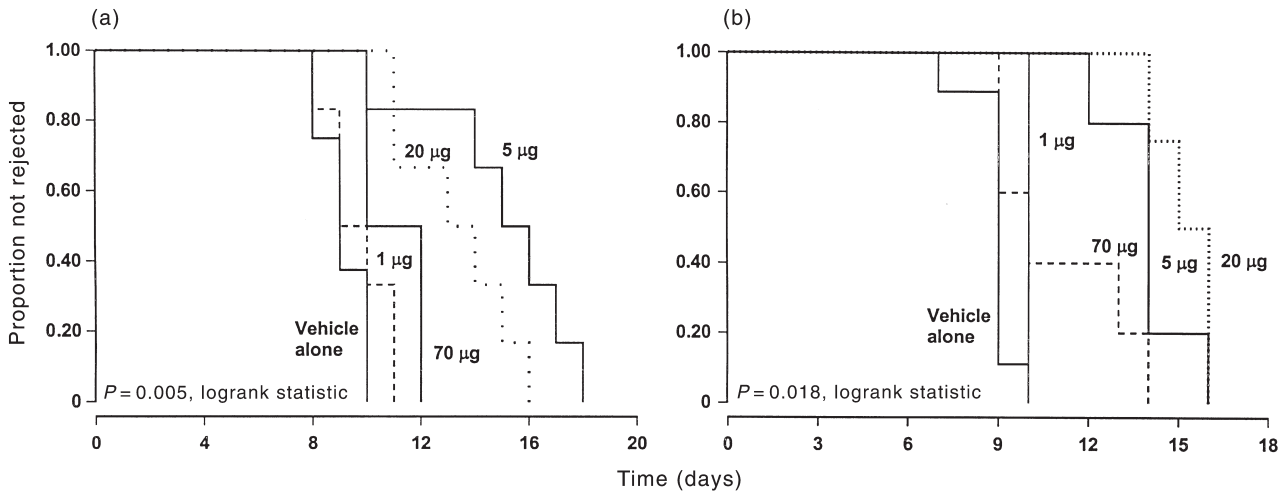

Figure 3 Skin grafts were exchanged between DA and Lewis rats. Significant prolongation of median time to rejection (TTR) was observed following delivery of recombinant human early pregnancy factor (rEPF) subcutaneously around the site of the graft. Full thickness abdominal skin was grafted on to a similar-sized defect on the lateral thoracic region and rats received twice daily injections of various doses of EPF around the graft site for 14 days. Day of rejection was that on which 50% of transplanted skin had undergone necrotic degradation. (a) DA skin grafted to Lewis rats. Time to rejection for vehicle alone, 9 days; 5 μg rEPF/dose, 15.5 days ($P = 0.02$); 20 μg/dose, 13.5 days ($P = 0.008$). (b) Lewis skin grafted to DA rats. Time to rejection for vehicle alone, 9 days; 5 μg rEPF/dose, 14 days ($P = 0.03$); 20 μg rEPF/dose, 15.5 days ($P = 0.008$). *P* values were determined by logrank statistics.

series of experiments, the most effective doses ranged between 5 and 20 μg rEPF/rat per 12 h. Median time to rejection (TTR) was 9 days with grafts receiving vehicle alone, while median TTR was 14 days in Lewis to DA grafts and 15.5 days in DA to Lewis grafts treated with doses of 5 μg ($P = 0.03$ and 0.02, respectively, logrank statistic). Median TTR was 13.5 days in DA to Lewis grafts and 15.5 days in Lewis to DA grafts treated with doses of 20 μg ($P = 0.008$ and 0.05, respectively, logrank statistic).

Discussion

Recombinant EPF was shown to be as active as nEPF and sEPF within the semiquantitative limits of the rosette inhibition test. Modification of the N-terminus of the recombinant molecule did not appear to affect its functional activity in this assay. N-peptide and the I-peptide gave similar results, demonstrating that there are a number of active sites within the molecule. The modified N-terminus did, however, affect the active half-life of the molecule in serum following i.p. injection. During early pregnancy, EPF is present in serum in multiple size forms. The large molecular weight form (≥ 250 kDa) apparently originates through association of smaller forms with a carrier protein or proteins.[4] The present studies suggest that the long half-life of nEPF and sEPF activity in serum is due to the ability of these molecules to bind to serum carrier proteins through the N-terminus and alterations to the N-terminal sequence of rEPF abrogate this binding. This suggestion is supported by the time course of activity of the EPF-derived peptides in serum. The N-terminal peptide has an active half-life in serum not significantly different from that of the native and synthetic molecules, while that of the I-peptide is similar to the recombinant molecule.

Treatment with EPF significantly prolonged viability of MHC class I and class II disparate allografts. The results demonstrated that in suppressing graft rejection, rEPF demonstrated a bell-shaped dose–response. This phenomenon has also been observed in the rosette inhibition test, with high as well as low doses giving a negative response.[18] The reason for this loss of responsiveness is not known, but it may reflect aggregation of the molecule above certain concentrations with consequent loss of biological activity. While insufficient recombinant material was available to continue EPF treatment beyond 14 days after grafting, the results presented here showed a significant prolongation of survival time in both the stringent models tested. This is the first report of the immunomodulatory activity of human EPF in suppressing graft rejection. Studies are now underway to test the effectiveness of the N-peptide in these models.

Because rEPF has a short half-life in serum, EPF was administered to the rats subcutaneously around the site of the graft.[19] This route of delivery would ensure that effective concentrations of rEPF were administered locally to control immunological events occurring at the graft site and in the draining lymph nodes. It is widely believed that donor dendritic cells (DC) sensitize the recipient to graft antigens and initiate the rejection process.[19] Donor-derived DC migrate from the graft into the recipient's spleen where they associate with and activate resting $CD4^+$ T cells. In addition, epidermal Langerhans cells (LC) migrate from allogeneic skin grafts, delivering donor LC into the recipient's nodes, providing a powerful stimulus for initiation of rejection.[20]

We have not yet studied the effect of EPF on cells of the DC lineage, but preliminary studies by Athanasas-Platsis (unpubl. data, 1999) have shown that a population (28%) of monocytes from peripheral human blood bind EPF. Other studies by Athanasas-Platsis and coworkers (unpubl. data,

1999) have shown that EPF suppresses Th1 responses. Early pregnancy factor binds to a subpopulation of $CD4^+$ T cells and, in so doing, suppresses IFN-γ production by activated cells. Partial characterization of cloned $CD4^+$ EPF-binding cells has shown that they are of the T helper/inducer phenotype. Allogeneic skin grafts are known to induce activation of Th1 cells with the production of IL-2 and IFN-γ and prolongation of graft survival is associated with downregulation of Th1-activated cells, with the immune response being driven towards a Th2 bias. We postulate that EPF prolongs the survival time of allogeneic skin grafts by downregulating immunostimulation of $CD4^+$ T cells. Whether it also has an effect on migration of antigen-presenting cells of the dendritic lineage has yet to be determined.

The recombinant EPF was effective in significantly prolonging the survival time of allogeneic skin grafts. However, in a standard lymphocyte proliferation assay *in vitro* and in the treatment of rats with experimental autoimmune encephalomyelitis *in vivo*, we have shown that rEPF is less active than sEPF (B Zhang *et al.*, unpubl. data, 1999). We are now preparing a recombinant molecule in insect cells, using the baculovirus expression system. This will produce an unmodified form of EPF including N-terminal acetylation and may provide a more appropriate molecule for further extensive studies in skin and organ transplant models.

Acknowledgement

Funding for this project was gratefully received from CSL Ltd, Australia.

References

1 Morton H, Hegh V, Clunie GJA. Studies of the rosette inhibition test in pregnant mice; evidence of immunosuppression? *Proc. R. Soc. Lond. B* 1976; **193**: 413–19.
2 Morton H. Early pregnancy factor: An extracellular chaperonin 10 homologue. *Immunol. Cell Biol.* 1998; **76**: 483–96.
3 Noonan FP, Halliday WJ, Morton H, Clunie GJA. Early pregnancy factor is immunosuppressive. *Nature* 1979; **278**: 649–51.
4 Rolfe BE, Morton H, Clarke FM. Early pregnancy factor is an immunosuppressive contaminant of commercial preparations of human chorionic gonadotrophin. *Clin. Exp. Immunol.* 1983; **51**: 45–52.
5 Rolfe BE, Cavanagh AC, Quinn KA, Morton H. Identification of two suppressor factors induced by early pregnancy factor. *Clin. Exp. Immunol.* 1988; **73**: 219–25.
6 Rolfe BE, Quinn KA, Athanasas-Platsis S, Cavanagh AC, Morton H. Genetically-restricted effector molecules released by human lymphocytes in response to early pregnancy factor. *Immunol. Cell Biol.* 1989; **67**: 205–8.
7 Athanasas-Platsis S, Quinn KA, Wong TY, Rolfe BE, Cavanagh AC, Morton H. Passive immunisation of pregnant mice against early pregnancy factor causes loss of embryonic viability. *J. Reprod. Fertil.* 1989; **87**: 495–502.
8 Athanasas-Platsis S, Corcoran CM, Kaye PL, Cavanagh AC, Morton H. Early pregnancy factor is required at two important stages of embryonic development in the mouse. *Am. J. Reprod. Immunol.* 2000; **43**: 223–33.
9 Quinn KA, Cavanagh AC, Hillyard NC, McKay DA, Morton H. Early pregnancy factor in liver regeneration after partial hepatectomy in rats: Relationship with chaperonon 10. *Hepatology* 1994; **20**: 1294–302.
10 Quinn KA, Athanasas-Platsis S, Wong T-Y, Rolfe BE, Cavanagh AC, Morton H. Monoclonal antibodies to early pregnancy factor perturb tumour cell growth. *Clin. Exp. Immunol.* 1990; **80**: 100–8.
11 Cavanagh AC, Morton H. The purification of early-pregnancy factor to homogeneity from human platelets and identification as chaperonin 10. *Eur. J. Biochem.* 1994; **222**: 551–60.
12 Hartman DJ, Hoogenraad NJ, Condron R, Hoj PB. Identification of a mammalian 10-kDa heat shock protein, a mitochondrial chaperonin 10 homologue essential for assisted folding of trimeric ornithine transcarbamoylase *in vitro. Proc. Natl Acad. Sci. USA* 1992; **89**: 3394–8.
13 Summers KM, Murphy RM, Webb GC *et al.* The human early pregnancy factor/chaperonin 10 gene family. *Biochem. Mol. Med.* 1996; **58**: 52–8.
14 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.
15 Love SG, Englebretsen DR, Garnham B *et al.* Observation of protein heptamer formation for chemically ligated early pregnancy factor. In: Ens W, Standing KG, Cherushevich IV (eds). *Nato ASI Series. Series C*, vol. 510. Dordrecht (Netherlands): Kluwer Academic Publishers, 1998; 171–9.
16 Somodevilla-Torres MJ, Hillyard NC, Morton H *et al.* Preparation and characterisation of polyclonal antibodies against human chaperonin 10. *Cell Stress Chaperones* 2000; **5**: 14–20.
17 Cavanagh AC, Morton H. EPF: Embryo detector and molecular chaperone. *Today's Life Sci.* 1996; **8**: 24–7.
18 Cavanagh AC. Production *in vitro* of mouse early pregnancy factor and purification to homogeneity. *J. Reprod. Fertil.* 1984; **71**: 581–92.
19 Gruber SA. The case for local immunosuppression. *Transplantation* 1992; **54**: 1–11.
20 Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM. Migration and maturation of Langerhans Cells in skin transplants and explants. *J. Exp. Med.* 1990; **172**: 1483–93.